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Thermosensitive Hydrogel as an In Situ Gelling Antimicrobial Ocular Dressing

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Abstract

Microbial keratitis is a severe ocular condition and one of the most prevalent causes of corneal scarring and associated blindness worldwide. Risk factors include contact lens use, ocular trauma, ocular surface disease and immunosuppression. Initial clinical management mandates intensive (hourly or more frequent) topical administration of broad spectrum antimicrobial therapy for at least 48 hours, which may require hospital admission, followed by tailored therapy based on microbiological investigation and the institution of strategies to reduce inflammation and promote healing. In this work we report an ocular wound dressing which can encapsulate and give sustained release of different antibiotics. The use of this dressing would allow patients to have eye drops on a 4 hourly basis, thereby facilitating treatment compliance and reducing hospital admissions.

Keywords: Wound dressing, hydrogel, antibiotic delivery, ocular drug delivery

Highlights:

- Chitosan/ β-glycerolphosphate gels can be used to incorporate antibiotics such as moxifloxacin and gentamicin.
- The antibiotics are released from the gels
- The gels are not toxic to ocular cells *in vitro*
- The antibiotic loaded gels can successfully kill bacteria both alone and in co-culture with human ocular cells.

1. Introduction

A healthy and intact ocular surface is critical for maintaining the transparency of the cornea and enabling clarity of vision. Infectious or non-infectious disease processes which compromise the ocular surface may lead to abnormalities in corneal structure, with loss of integrity of the normal parallel alignment of collagen fibrils resulting in severe visual impairment. Corneal infection (microbial keratitis) rarely occurs in the healthy human eye due to complex host defence mechanism[1]. However, interruption of the corneal surface epithelium and/or an abnormal ocular tear film allows the introduction of micro-organisms, such as bacteria, fungi and protozoa, into the corneal stroma, where they are able to proliferate and cause inflammation and toxin release that contributes to destruction of the corneal substance[2]. Microbial keratitis is a serious ocular condition, and presents a significant disease burden that may result in severe visual disability[3]. Bacterial infection is the most common cause of infection with contact lens wear the biggest predisposing factor to corneal infection, although other risk factors are also associated, including recent ocular surgery, trauma, ocular surface disease, lid margin abnormalities, impaired corneal sensation, chronic use of topical steroids and systemic conditions such as diabetes mellitus, vitamin A deficiency and immunocompromised states[3]. Bacterial keratitis requires prompt treatment with intensive antimicrobial therapy to prevent sight threatening complications, such as scarring, corneal perforation and endophthalmitis[2]. Aims of treatment include initial rapid sterilisation of the infective pathogen, followed by reduction in the inflammatory response, prevention of corneal scarring, and facilitation of epithelial healing[4]. The avascular corneal tissue precludes effective local response from systemic administration of antimicrobial agents, and thus the initial sterilisation phase of treatment typically consists of frequent (hourly or more frequent) topical antibiotics[5]; hospital admission may be necessary where treatment compliance is unlikely or assistance is required with intensive topical therapy. Intensity of topical administration can then be tapered according to clinical response. As laboratory isolation of the infective pathogen may take some time, initial broad spectrum empirical antibiotic therapy is instituted to cover both Gram positive and Gram negative organisms. Topical fluoroquinolones (e.g. ofloxacin, levofloxacin and moxifloxacin) are both well tolerated and effective as monotherapy. Alternatively, dual antimicrobial therapy with fortified cephalosporin, such as cefuroxime 5%, and aminoglycoside (e.g. gentamicin 1.5%) are also effective[6]. Moreover, prolonged use of aminoglycoside may delay epithelial repair or cause local ocular surface to fluoroquinolone monotherapy may be more effective than dual therapy and is better tolerated[7,8,3]. Moxifloxacin is a fourth generation fluoroquinolone antibiotic, with broad-spectrum activity against Gram negative and Gram positive bacteria. The mechanism of action of moxifloxacin involves inhibition of DNA gyrase and topoisomerase IV enzymes required for the replication, transcription and repair of bacterial DNA[9,10,11]. In *S. pneumoniae*, it has been shown that moxifloxacin is a poor substrate for active efflux, hence its efflux from bacterial cells is reduced[12], therefore increasing its antibacterial potency, it has also been shown to have lower MIC than fortified antibiotics against ocular pathogens[12]. Gentamicin is an aminoglycoside which inhibits bacterial growth by targeting the bacterial ribosome. It is a broad spectrum antibiotic and is especially effective against Gram positive *Staphylococcus* and Gram negative bacteria. Although effective, one of the major challenges in topically administered drugs is that they are cleaved and washed away rapidly, that is they remain on the cornea for less than two minutes, thus reducing their efficacy and needing frequent administrations by the patient on a daily basis. To enhance the efficacy of moxifloxacin on the corneal surface, various drug delivery systems such as hydrogel based gels have been evaluated.

Gellan, alginate, karageenan and chitosan for example have shown to tailor the release of moxifloxacin over a longer time period and therefore reducing the need for frequent administration of the drug.

Chitosan is an aminopolysaccharide derived from the partial depolymerisation and deacetylation of chitin, a component found in the exoskeletons of crustacean shells[13]. Chitosan has been proven to be advantageous for medical applications due to its biocompatibility, biodegradability and low cytotoxicity, and has received much attention as the basis of a drug delivery system[14]. In 2000, Chenite et al. developed an injectable, thermosensitive, pH-dependent solution based on the neutralisation of chitosan by addition of β-glycerolphosphate[15]. The resulting solution is a reversibly thermosensitive polymer which is a liquid at room temperature, but a gel at 37°C[16]. Thermoresponsive materials have gained increasing importance in potential treatments. These are usually formed from co-block polymers[17,18]. These materials are then used to incorporate therapeutics which can be released *in situ*[19,20]. Further developments in this field have moved towards utilising these polymer systems as smart materials where they can be tuned to respond to multiple stimuli or can act as a reporting device *in situ*[21,22]. Loh *et al* have previously reported the use of a thermogelling system to deliver natamycin to the eye using poly(F127/PTHF urethane) hydrogels[23]. The thermosensitivity of the chitosan β-glycerolphosphate system is biomedically relevant in wound healing as the liquid can be applied to the front of the eye and flow over the complex topography of the wounded eye before the transforming into a protective, transparent, gel wound dressing[24].

The aim of this work is to develop a flowable wound dressing for the eye which can be administered as a liquid eye drop but forms a firm gel dressing on contact with the cornea. The gel has antibacterial drug, such as moxifloxacin or gentamicin, incorporated to eliminate bacteria which have colonised the wound and also acts as an occlusive dressing to protect the cornea.

2. Materials and Methods

All materials used in this project were purchased from Sigma (Poole, UK) unless stated otherwise.

2.1 Bacterial strains.

The bacteria used in this study were clinical isolates from patients at the Queen Elizabeth Hospital, Birmingham, UK;

2.2 Hydrogel Preparation

This was carried out as previously described by Chenite et al[15]. Briefly, chitosan was dissolved in (0.1 M) HCl solution using a magnetic stirrer for 3 hours. The resulting solution was then chilled at 4°C for 3 hours. β-glycerolphosphate dissolved in deionised water was added drop-wise to the chilled chitosan solution whilst stirring to produce a clear, homogenous liquid solution. The solution was incubated at 37 °C to form a gel. For antibiotic loaded samples, moxifloxacin (100 µL, 0.25 µM) or gentamicin (100 µL, 1 % v/v) were added to the Chitosan/β-glycerolphosphate liquid solution, vortexed for 30 seconds and incubated at 37 °C to form a gel.

2.3 Rheological characterisation

A parallel plate rheometer (TA instruments ARES system) was used to characterise the gelation process. The plate diameter was 35 mm and the frequency was 1 Hz. Chitosan (Heppe-Medical Chitosan, Halle, Germany) / β-glycerolphosphate solution was prepared as previously described. Temperature scans were performed on the hydrogel to characterise the thermosensitive gelation process, gelation was indicated by an increase in the storage modulus (G').

2.4 Antimicrobial efficacy testing of the hydrogel

The Chitosan/ β-glycerolphosphate gels with and without moxifloxacin and gentamicin were prepared as previously described and inoculated with *S. aureus* from an overnight culture in Lennox Broth (LB) broth (10 µL, ~1x10^6 cells). Six gels were prepared per treatment and set in a 24 well plate at 37 °C and LB Broth added to the well. The gels were incubated overnight at 37 °C. The gels were removed from the incubator and the broth removed. The gels were vortexed to break up the structure and serially diluted in sterile phosphate buffered saline (PBS) and then plated out on LB Agar. The plates were incubated overnight at 37 °C and the visible colony forming units counted.

2.5 Release of moxifloxacin and gentamicin from chitosan/ β-glycerolphosphate gels

Chitosan/ β-glycerolphosphate gels were loaded with antibiotics, moxifloxacin (100 µL, 0.25 µM) and gentamicin (100 µL, 1 % v/v) and gelled at 37°C. The gels were suspended in PBS (1 mL). At each time point the PBS was removed and fresh PBS added to the vial. The release of moxifloxacin was measured using absorbance at 293 nm according to literature procedures[25]. The release of gentamicin was measured using absorbance at 202 nm according to literature procedures[26]. The cumulative release was obtained by the summation of the previous absorbance values.

2.6 Antibacterial efficacy of released antibiotics

The solutions obtained from the release study were then tested for antimicrobial efficacy. *S. aureus* was grown overnight in LB Broth. The solutions were then diluted in LB Broth (1:1) and inoculated with *S. aureus* followed by incubation overnight at 37 °C. The solutions were serially diluted with sterile water and plated out on LB agar and the plates incubated at 37 °C overnight. The number of colonies on the plate were counted.

2.7 Cytotoxicity of gels to primary corneal fibroblast cells.

Primary human corneal fibroblast cells were isolated from donor human corneas under ethics (08/H1206/165). The cells were cultured in DMEM substituted with fetal calf serum (10 % v/v) and penicillin/streptomycin (1 % v/v). Cells were harvested at confluency by removal of the media and the cell monolayer washed three times with PBS (5 mL) and then incubated in trypsin:EDTA (2 mL) for 5 minutes at 37 °C. Once the cells were detached from the culture flask the trypsin:EDTA was blocked using cell media and the cells seeded into a 24 well plate at a density of 50,000 cells/ well and 6 wells/ treatment group. The cells were cultured to confluency in the well, the media was removed and the cells washed 5 times to remove and residual penicillin/streptomycin. Fresh media with no penicillin

was added to the cells and the gels added on top of the cell monolayer. The cultures were incubated at 37 °C overnight and the cell viability measured using established protocols for the alamar blue metabolic assay[27].

2.8 Efficacy of antibiotic loaded gels in a co-culture of human corneal fibroblasts and S. aureus.

Cells were seeded and plated as described above. Once the cells reached confluence, the cell media was removed and the cell monolayer was washed 5 times with PBS to remove and residual penicillin/streptomycin. Fresh DMEM substituted only with fetal calf serum (10 %) was added to each well (1 mL), the wells were then inoculated with *S. aureus* in LB broth (10 µl, ~100,000 cells). Gels were then added to each of the wells in the following treatment groups: 1) Cell monolayer with bacteria (Untreated), 2) Cell monolayer with bacteria and an unloaded gel (Chitosan), 3) Cell monolayer with bacteria and a gel loaded with gentamicin (Chitosan + Gentamicin), 4) Cell monolayer with bacteria and a gel loaded with moxifloxacin (Chitosan + Moxifloxacin), 5) Cell monolayer with bacteria and gentamicin added directly to the cell media, no gel present (Gentamicin 5 µL, 1 % v/v), 6) Cell monolayer with bacteria and moxifloxacin (5 µL, 0.005 µM)added directly to the cell media, no gel present (Moxifloxacin). All the treatments were incubated overnight at 37 °C. The media and the gels were removed from the system and vortexed to break up the structure and the cell monolayer washed with PBS. The media, PBS wash and gel were mixed and serially diluted using sterile water before being plated on LB agar to determine the bacteria levels. The cell monolayer was washed a further 3 times and incubated in trypsin:EDTA (250 µL) at 37 °C for 5 minutes and the cells counted using a haemocytometer.

2.9 Statistical Analysis

All statistical analysis was carried out using SPSS 17.0 (IBM SPSS Inc., Chicago, IL) and data was presented as mean ± SEM. The Shapiro-Wilk test was used to ensure all data was normally distributed before parametric testing using a one-way ANOVA with Tukey post-hoc test. Statistical significance threshold was $p<0.05$.

3. Results

3.1 Thermal gelation of Chitosan/ β-glycerolphosphate gels

Chitosan (500 kDa) with a 95 % degree of deactylation showed thermosensitivity when mixed with β-glycerol phosphate. At temperatures < 29°C the chitosan exists as a stable viscous liquid. At 29°C the sol-gel transition initiates and the G' of the solution increases over 9°C until it plateaus at 38°C when full gelation had occurred (Figure 1).

3.2 Antibacterial Efficacy of the Gels

The gels were tested for antibacterial efficacy against *S. aureus*, both antibiotics could significantly inhibit the bacteria growth with no colony forming units seen after culturing bacteria with

moxifloxacin or gentamicin (Figure 2). When the antibiotics were loaded into the gels, the same inhibition was seen and no colony forming units were observed. However, when unloaded gels were used bacterial colony forming units (cfu) of 421 ± 116 and 380 ± 84 as the gel could not inhibit bacterial growth alone. These were significantly higher than antibiotic loaded gels ($p<0.000$) and not significantly different from controls 292 ± 130 cfu and 362 ± 89 cfu.

3.3 Release of Antibiotics from the Gel

The gels show a sustained cumulative release of both antibiotics over the 4 hour period (Figure 3a). At 30 minutes both antibiotic loaded gels showed significantly higher absorbance unit (a.u.) values than control (0.09 ± 0.01 a.u.) with moxifloxacin and gentamicin displaying values of (1.53 ± 0.01 a.u.) and (0.52 ± 0.04 a.u.) respectively. The absorbance readings for both antibiotic loaded gels increase over the 4 hour period finishing at 6.18 ± 0.36 a.u. (moxifloxacin) and 1.22 ± 0.01 a.u. (gentamicin) both significantly higher than control levels ($p<0.00$). The antimicrobial efficacy of the solutions were then tested against *S. aureus* (Figure 3b). This showed that at 0.5 hours, both antibiotic loaded gels had released enough antibiotic to impact bacterial survival. Bacterial colonies grew on gentamicin loaded gels (2.44 ± 2.1 cfu) and on moxifloxacin loaded gels (0.66 ± 0.70 cfu). Although this was not complete inhibition, it was significantly lower than unloaded gels (1121 ± 575 cfu). At all later times complete inhibition was observed for both antibiotics at release solutions 0.5,1,2 and 4 hours, unlike unloaded gels which showed bacterial counts at 1 hour (653 ± 301 cfu), 2 hours (1233 ± 777 cfu) and 4 hours (182 ± 34 cfu).

3.4 Cytotoxicity of chitosan gels

The gels were tested for toxicology in corneal fibroblast cells (Figure 4). The number of surviving cells following incubation with both antibiotic loaded and unloaded gels for 24 hours was measured. The alamar blue assay (linked to cell number) showed no differences between the cells exposed to the gel and cells which had not been exposed to the gel. The antibiotics also showed no cytotoxic effects on the cells.

3.5 Efficacy and toxicity in bacteria-fibroblast co-cultures.

The bacteria number and cell number in the co-culture were recorded to determine the toxicity and antibacterial efficacy (Figure 5a-b). The data showed that without treatment there was no inhibition of bacterial growth ($1.9 \times 10^6 \pm 8.6 \times 10^5$ cfu) but there had been a significant reduction in corneal fibroblast cell number ($7.8 \times 10^4 \pm 4.7 \times 10^3$ cells /mL) compared to the other treatment groups ($p<0.001$). In cultures with the unloaded gel some inhibition of bacterial growth was observed with a significant reduction to ($1.7 \times 10^5 \pm 7.5 \times 10^4$ cfu). This also showed a corresponding increase in the number of corneal fibroblasts cells surviving ($1.2 \times 10^5 \pm 3.6 \times 10^3$ cells/mL), which was not significantly different from the other treatment groups. Both antibiotic loaded gels significantly reduced bacterial growth with moxifloxacin loaded gel treated wells showing 1.88 ± 0.87 colony forming units and gentamicin loaded gel treated wells showing 2.22 ± 1.28 colony forming units. These are both

significantly lower than control samples ($p<0.000$). These also showed significantly higher corneal fibroblast cell numbers than control ($1.2 \times 10^5 \pm 1.3 \times 10^4$ and $1.1 \times 10^5 \pm 1.4 \times 10^4$ gentamicin loaded gel and moxifloxacin loaded gel, respectively). Finally, both gentamicin and moxifloxacin showed complete inhibition of bacterial growth with no colony forming units seen, significantly lower than controls, but not significantly lower than the antibiotic loaded gels. They also demonstrated higher cell growth than controls with moxifloxacin treated cells showing a cell number of $1.2 \times 10^5 \pm 1.7 \times 10^4$ and gentamicin treated wells showing $1.1 \times 10^5 \pm 1.0 \times 10^4$.

4. Discussion

The treatment of bacterial keratitis using topical administration of antibiotics is well established[5,6]. However, the delivery of the eye drops, every 15 minutes for up to 48 hours represents a significant problem to the health and wellbeing of the patients[4]. Accordingly this study has investigated the use of hydrogels to deliver the antibiotics to the eye over time, while also acting as an occlusive dressing protecting the damaged cornea. Here we have demonstrated the release, antibacterial efficacy and low *in vitro* toxicity of the antibiotic loaded chitosan/β-glycerolphosphate gels.

Chitosan is a polymeric material with the ability to form a thermosensitive, hydrogel which is a viscous liquid at room and a gel at body temperature. This unique ability makes it a suitable biomaterial for application as wound dressing[28]. Rheological temperature sweep indicates that the chitosan β-glycerolphosphate solution can be gelled at 38°C. Subsequent to this the gel existed as a stable viscous liquid at temperatures < 29 °C, while sol/gel transition temperature ranged from 29 – 37°C. This was similar to studies by Cheng et al.,[29] where gelation temperature of chitosan/β glycerol phosphate was found to be at 37°C. As was suggested by Cho et al.,[16] the main molecular forces responsible for the sol/gel formation include electrostatic force, hydrogen bonding and electrostatic force interactions between chitosan chains and β-glycerolphosphate.

Due to its position as an external surface, the temperature of the cornea can be affected by the body core temperature, the ambient temperature, as well as changes in blinking rate. In humans, corneal temperature has been found to peak at 37 ºC regardless of any increases in body temperature[30], but reaches a maximum of 36.5 - 37ºC when ambient temperature is between 32.0 - 34.5ºC. The association between changes in corneal temperature and the occurrence of eye diseases has been identified[31,32]. From our experiments, the ability of the gel to attain gel consistency at 37ºC will be advantageous for application since the cornea is at this same temperature irrespective of ambient or body heat.

There are been several reports that highlight chitosan's intrinsic antimicrobial properties. These properties are associated with the ability of chitosan to destabilise the outer membrane of Gram negative bacteria and permeabilise other microbial membranes. No et al.,[33] showed that chitosan had greater antimicrobial effects against Gram positive bacteria than Gram negative bacteria and when at lower pH. Further, Raafat et al.,[34] demonstrated that the antimicrobial efficacy of chitosan is not dependent on a single target but that chitosan binds to teichoic acids, leading to extraction of membrane lipids, cell lysis and eventually bacterial cell death[35]. They showed the occurrence of multiple changes transcriptional expression of genes responsible for regulation of stress, autolysis and energy metabolism in *Staphylococcus aureus*, when bacteria are treated with chitosan. In our study

we compared the antimicrobial efficacy of chitosan hydrogel with and without an antibiotic loading. Accordingly, we report that chitosan based gels can decrease bacterial load. However, to obtain complete bacterial inhibition the gels require antibacterial agents to be incorporated.

In antibiotic loaded hydrogels, we observed inhibition of viability *S. aureus* which was successfully inhibited by moxifloxacin and gentamicin released from the gels, as no viable colonies were retrieved. Similarly, work by Nayak and colleagues[36] investigating the antimicrobial activities of moxifloxacin loaded hydrogel formulations consisting polyacrylic acid (Carbopol 934) and propyl methyl cellulose (k15M), showed inhibition on agar plates with *S. aureus*.

In the release assays, our data showed that moxifloxacin and gentamicin were successfully and steadily released from the chitosan gels over a 4 hour time period. Release of the antibiotics did not follow a slow, time dependent course, however, showed a sustained release of drug over time as previously observed by Nanjwade et al.,[37]. We determined the concentration of moxifloxacin released at 4 hours compared to stock concentrations was approximately 0.6mg/ml released from gels containing. This result is comparable with Bajgrowicz et al., who showed the concentration of moxifloxacin released form daily disposable contact lenses was up to 0.2 mg/ml[38].

Chitosan based materials have been reported extensively in the literature for medical applications especially wound dressing[39,40,41]. Similarly, to literature sources, in tissue culture, we report no toxicity from the chitosan based gels. Moxifloxacin and gentamicin are MHRA approved for the treatment of microbial keratitis and as such are not expected to induce a toxic effect in corneal cells[42,43]. This supports our data that neither antibiotic is toxic to the cells. In co-culture experiments the data showed a significant decrease in cell survival in the untreated control. This shows the demonstrated *in vitro* the ability of the bacteria to affect the corneal cells. The presence of the antibiotic loaded gels had the same effect as antibiotic drops at inhibiting the growth of the bacteria and producing a more permissive environment for corneal cell growth. This supports the use of the hydrogel as inclusion of the antibiotics in the gel does not hinder their efficacy.

5. Conclusions

In summary, the hydrogels exist as a chitosan/β-glycerolphosphate solution which can be administered as an eye drop that will form a transparent dressing on the front of the eye. The dressing will deliver a sustained release of antibiotics killing bacteria, but not harming the cells in the cornea. This will ensure that the patient could be treated at much longer time points, meaning that patients could be treated as outpatients, reducing hospital admissions.

Acknowledgments

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Figure Legends

Figure 1 Variation of G' and G" with temperature showing the gelation of the chitosan/β-Glycerol phosphate blend, n=3, error bars show standard error of the mean.

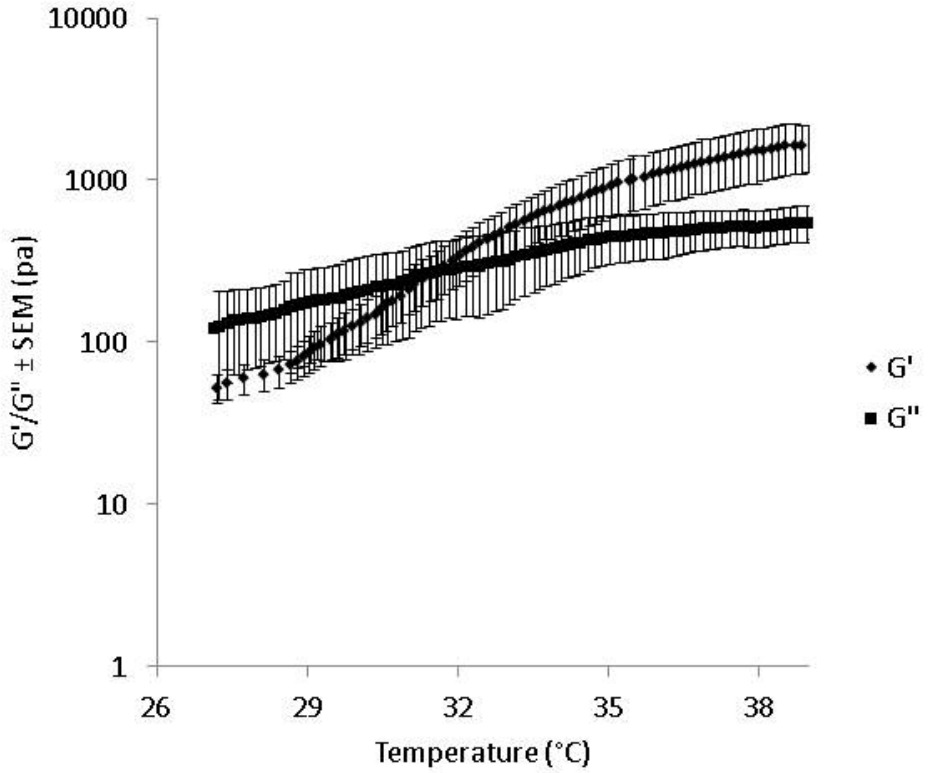
Figure 2 Antimicrobial activity of chitosan gels with and without antibiotics moxifloxacin/gentamicin, n=6, error bars show standard error of the mean, *** denotes statistical significance <0.000

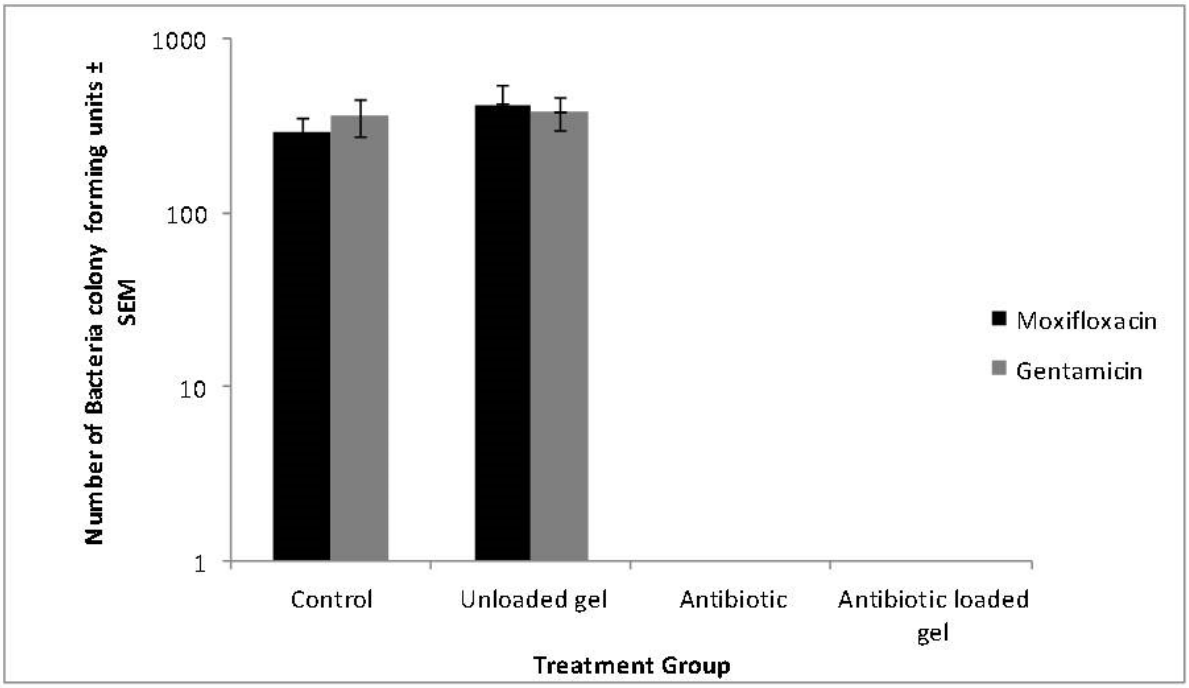
Figure 3 a) Release of moxifloxacin and gentamicin from the hydrogel over 4 hour period, n=3, error bars show standard error of the mean. b) antibacterial efficacy testing of the solutions from the release studies at each time point.

Figure 4 Cytotoxicity of chitosan/β-glycerol phosphate gels, chitosan/β-glycerol phosphate gels with antibiotics and antibiotics in solution. Analysed using resazurin, with values shown representing averages of three biological replicates, p>0.05

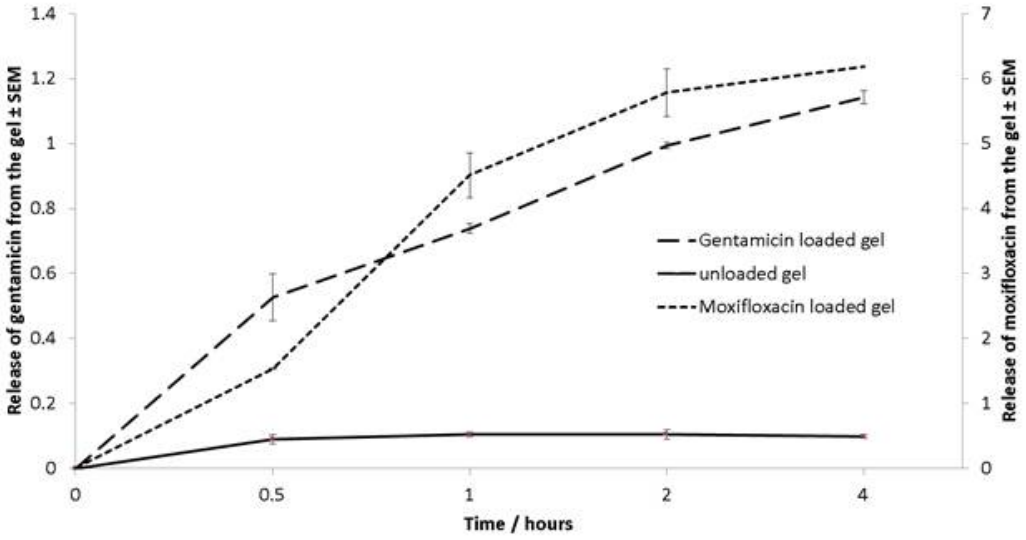
Figure 5 Cytotoxicity and bacterial efficacy in co-cultures of human corneal fibroblasts and S. aureus. a) Cytotoxicity of bacterial co-cultures with chitosan/β-glycerolphosphate gel with and without antibiotics. b) Bacterial efficacy of chitosan/β-glycerolphosphate gel with and without antibiotics in co-culture with human corneal fibroblasts. Values shown representing averages of three biological replicates, p>0.05

a)

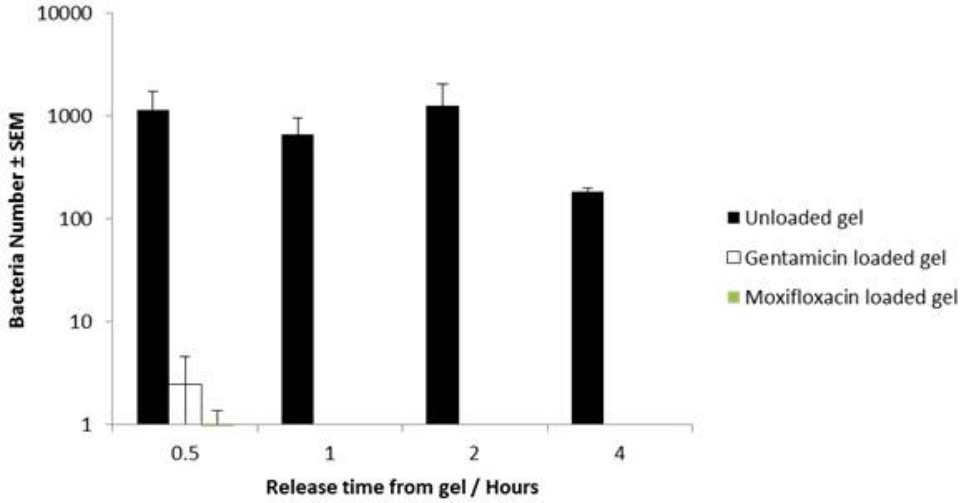


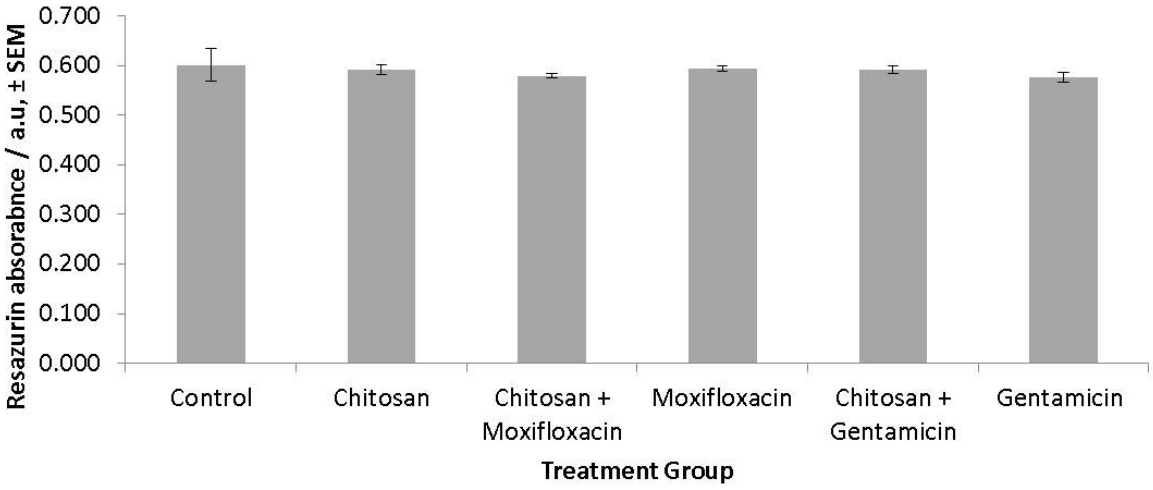

1000
100
10
1
Number of Bacteria colony forming units ± SEM
Moxifloxacin
Gentamicin
Control
Unloaded gel
Antibiotic
Antibiotic loaded gel
Treatment Group

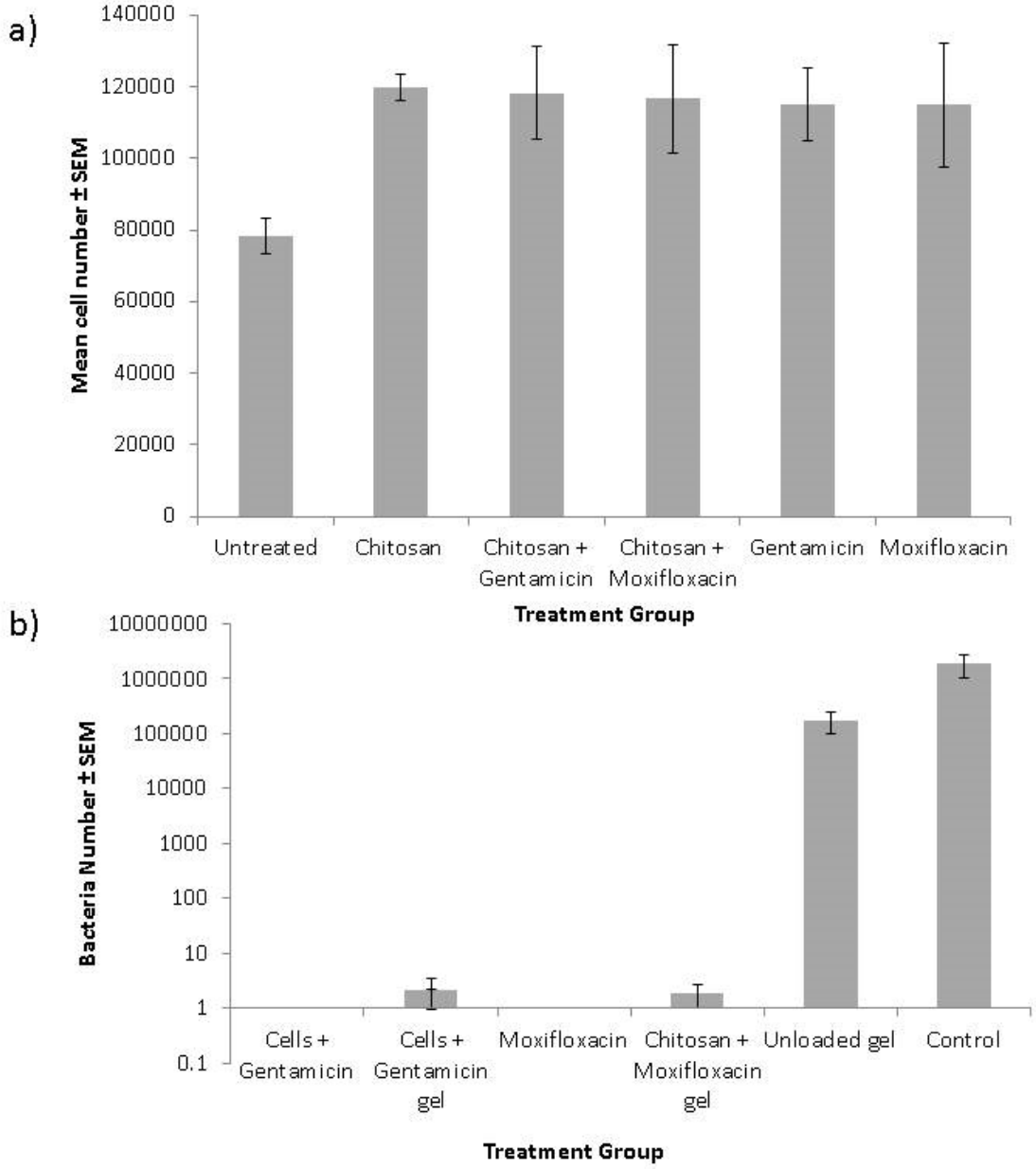
a)


Release of gentamicin from the gel ± SEM
Release of moxifloxacin from the gel ± SEM
Time / hours
Gentamicin loaded gel
unloaded gel
Moxifloxacin loaded gel

b)


Bacteria Number ± SEM
Release time from gel / Hours
Unloaded gel
Gentamicin loaded gel
Moxifloxacin loaded gel


Resazurin absorabnce / a.u, ± SEM
0.700
0.600
0.500
0.400
0.300
0.200
0.100
0.000
Control
Chitosan
Chitosan + Moxifloxacin
Moxifloxacin
Chitosan + Gentamicin
Gentamicin
Treatment Group



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